

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
6 September 2002 (06.09.2002)

PCT

(10) International Publication Number
WO 02/067899 A1

- (51) International Patent Classification⁷: A61K 9/107, 47/14, 39/39, B01F 17/34 (74) Agent: VAN GENT, M.; P.O. Box 31, NL-5830 AA Boxmeer (NL).
- (21) International Application Number: PCT/EP02/02145 (81) Designated States (*national*): AE, AG, AL, AU, BA, BB, BG, BR, BZ, CA, CN, CO, CR, CU, CZ, DM, DZ, EC, EE, GD, GE, HR, HU, ID, IL, IN, IS, JP, KP, KR, LC, LK, LR, LT, LV, MA, MD, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SD, SG, SI, SK, TR, TT, UA, US, UZ, VN, YU, ZA.
- (22) International Filing Date: 26 February 2002 (26.02.2002)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data: 01200745.6 28 February 2001 (28.02.2001) EP (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- (72) Inventors; and (75) Inventors/Applicants (*for US only*): JANSEN, Theodorus [NL/NL]; Wilhelminastraat 15, NL-5802 BA Venray (NL). SCHIJNS, Virgil, Elisabeth, Joseph, Caspar [NL/NL]; Albert van Dalsumlaan 53, NL-3584 HA Utrecht (NL). HERMKENS, Erik [NL/NL]; O.L. Vrouwenstraat 19, NL-5423 SH Handel (NL).
- Published:
— with international search report
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/067899 A1

(54) Title: INJECTABLE WATER-IN-OIL EMULSIONS

(57) Abstract: The present invention provides adjuvants, for use in vaccine formulation, comprising a water-in-oil emulsion, characterised in that said emulsion comprises a polymeric emulsifier which is a block copolymer having a general formula A-COO-B-OOC-A, in which B is the divalent residue of a water-soluble polyalkylene glycol and A is the residue of a oil-soluble complex mono-carboxylic acid. Such polymeric emulsifier, as well as the preparation thereof, has been disclosed in GB 2002400 and WO9607689. Preferably in the blockcopolymers of formula A-COO-B-OOC-A, component B is derived from polyethylene glycol and components A are derived from polyhydroxystearic acid, preferably from poly(12-hydroxy-stearic acid). Most preferred emulsifiers used in the adjuvants according to the invention are Arlacel P135, a PEG 30 Dipolyhydroxystearate. Another, similar, emulsifier for use with the invention is ATLOX[®]4912. Both Arlacel P135 and ATLOX[®]4912 are block copolymers (A-B-A) of polyethylene glycol and hydroxystearic acid with a molweight of approximately 5000 commercially available from ICI. It has been found that with the use of these emulsifiers adjuvants based on w/o emulsions that are stable and have a low viscosity can be obtained that may be based on metabolisable oils. Said adjuvants have an excellent adjuvant activity, and are well tolerated.

Injectable water-in-oil emulsions

The present invention relates to adjuvants comprising water-in-oil emulsions, a method to prepare these emulsions, and vaccines comprising the adjuvants.

Water-in-oil (w/o) emulsions are two phase systems consisting of a continuous oil phase and an aqueous phase (discontinuous phase) whereby the aqueous phase is dispersed as small droplets in the oil phase, and one or more surfactants and emulsifiers. W/o emulsions are widely applied in medicine, cosmetics and the food and beverage industry. In medicine w/o emulsions are generally used in pharmaceutical formulations as vehicle of therapeutic agents, especially in case of water-insoluble or water-sensitive active ingredients. In vaccination, w/o emulsions are commonly used as adjuvant to stimulate the immune response against target antigens derived from one or more infectious agent(s). One of the oldest w/o emulsion adjuvant is Freund's Complete Adjuvant (FCA) containing mycobacteria in mineral oil and Arlacel A® as surfactant or Freund's Incomplete Adjuvant, which lacks the mycobacteria. Other w/o emulsions of mineral or metabolisable oils have been developed and are regularly used as adjuvant in vaccination.

W/o emulsions are generally applied via injection. To be injectable a composition must be substantially fluid. However the w/o emulsions are often relatively viscous which makes injection of these emulsions very difficult. Especially with w/o emulsions of metabolisable oils the viscosity is a problem.

In w/o emulsions, the viscosity in general is dependent on the viscosity of the continuous phase, that is the oil phase.

Different oils can be used in adjuvants, both mineral and non-mineral (metabolisable) oils. However, although oil based adjuvants generally increase the immunological activity of the vaccines, compared to non-oily vaccines, they can cause local reactions at the injection sites of the vaccines, especially when mineral oils are used. This may be caused by the fact that mineral oils cannot be metabolised by the vaccinated organism and tend to stay close to the injection sites.

In view of the problem of local reactions at the injection site, the replacement of mineral oils by non-mineral, metabolisable, oils would be desirable. However, the immunogenic activity of the adjuvant should remain high, when using the mineral oil instead of the non-mineral oil. Preferably, the immunogenic activity of adjuvants based on metabolisable oils should be as high as for the adjuvants based on mineral oils, while the problem of local tolerance is virtually absent.

CONFIRMATION COPY

Moreover, the adjuvants based on w/o emulsion on the basis of non-mineral oil should be stable, and should have an acceptable shelf life.

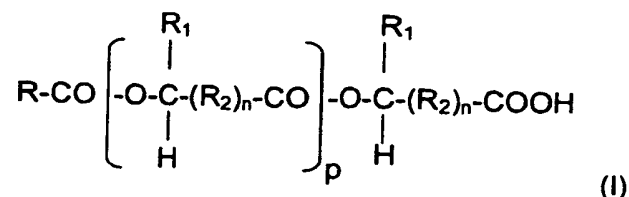
However, metabolisable oils, and in particular (semi-) synthetic- and vegetable oils are viscous at room temperature and their use in w/o emulsions leads to emulsion viscosity's that are similar to that of the individual oil. A reduction of the oil content (and consequently an increase in the water content) however often causes an increase in the emulsion viscosity to such an extent that injection is no longer possible. Besides an effect on the viscosity, changes in the oil content of a w/o emulsion effect the stability of the emulsion. Reduction of the oil content results in an enlarged interfacial area. The quantity of emulsifier (dependant on the size of the interfacial area) will be insufficient and the emulsion will break.

The size of the interfacial area is also dependant on the droplet size of the dispersed phase: a reduced droplet size results in an enlarged interfacial area. Mechanical means can be used to reduce or increase the droplet size of the dispersed phase. Compared to high shear processing equipment, low shear processing results in increased droplet size and, therefore, reduced interfacial area.

These limitations make it difficult to obtain stable and fluid w/o emulsions, especially when based on metabolisable oils. Hence there is a need to find other methods and/or means to obtain stable w/o emulsions, which at the same time are fluid. The present invention aims to provide adjuvants based on stable w/o emulsions that are very suitable for injection, even when metabolisable oils are used, and have a good adjuvant activity.

Surprisingly it has been found that when certain specific emulsifiers are used w/o emulsions can be made that are stable, and provide excellent adjuvant activity, even when based on non-mineral, metabolisable oils. The emulsions have a very low viscosity and thus suitable for injection.

The present invention therefore provides adjuvant, for use in vaccine formulation, comprising a water-in-oil emulsion, characterised in that said emulsion comprises a polymeric emulsifier which is a block copolymer having a general formula A-COO-B-OOC-A, in which B is the divalent residue of a water-soluble polyalkylene glycol and A is the residue of an oil-soluble complex monocarboxylic acid. Such polymeric emulsifiers, as well as the preparation thereof, have been disclosed in GB 2002400 and WO9607689, the contents of which are herewith incorporated by reference. The emulsifiers, as described in GB 2002400, are emulsifiers wherein A has a molecular weight of at least 500 and is the residue of an oil-soluble complex mononcarboxylic acid, i.e. a fatty acid. These complex monocarboxylic acids may be represented by the general formula:



in which

R is hydrogen or a monovalent hydrocarbon or substituted hydrocarbon group;

R₁ is hydrogen or a monovalent C₁ to C₂₄ hydrocarbon group;

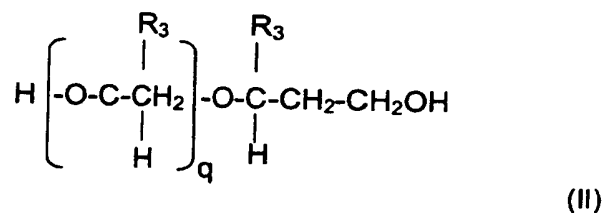
R₂ is a divalent C₁ to C₂₄ hydrocarbon group;

n is zero or 1;

p is an integer from zero to 200.

The units between the brackets in formula 1 may be all the same or they may differ in respect of R₁, R₂ and n. The quantity p will not normally have the same unique value for all molecules of the complex acid but will be statistically distributed about an average value lying within the range stated, as is commonplace in polymeric materials.

Polymeric component B has a molecular weight of at least 500 and is the divalent residue of a water-soluble polyalkylene glycol having the general formula



wherein

R₃ is hydrogen or a C₁ to C₃ alkyl group;

q is an integer from 10 up to 500.

The repetitive units in formula II again may all be the same or may differ in R₃, and the quantity q may vary about an average value.

The hydrocarbons R, R₁, and R₂ may be linear or branched.

Preferably in the block copolymers of formula A-COO-B-OOC-A, component B is derived from polyethylene glycol and components A are derived from stearic acid, for example polyhydroxystearic acid, preferably from poly (12-hydroxy-stearic acid).

Thus, R may be a straight chain C₁₇H₃₅-group derived from stearic acid, and the unit containing R₁ and R₂ may be derived from 12-hydroxy-stearic acid. p, in this case, preferably has a value of at least 2.

CONFIRMATION COPY

Preferably q may have a value between 20 and 60, more preferably above 23.

The weight ratio of the combined components A to the component B may vary widely, and typically will lie in the range from 9:1 to 1:9.

Most preferred emulsifiers used in the adjuvants according to the invention are Arlacel P135, a PEG 30 Dipolyhydroxystearate. Another, similar, emulsifier for use with the invention is ATLOX®4912. Both Arlacel P135 and ATLOX®4912 are block copolymers (A-B-A) of polyethylene glycol and polyhydroxystearic acid with a mol weight of approximately 5000 commercially available from ICI.

These polymeric block copolymers were found to be compatible with a wide variety of oils, thus providing a much wider range of w/o emulsions having the required stability and fluidity to ensure administration via injection, and which are very well tolerated by the vaccinated subjects. Furthermore the use of these ABA type block copolymers lead to w/o emulsions having excellent stability during storage thus improving the shelf life of said emulsions. The resulting w/o emulsions were stable and fluid at low temperatures, especially at 25°C.

Most important, the adjuvants according to the invention, based on the use of the above-mentioned ABA block copolymer type emulsifiers surprisingly had excellent immunostimulating (adjuvant) activity when used in vaccines, while no local reactions at the injection site were induced.

The w/o emulsions according to the invention may comprise 0.01 – 15% w/w, preferably 0.05 – 10% w/w, more preferably 0.1 – 3% w/w, most preferably 0.3-0.5% w/w of the polymeric emulsifier according to the invention. In a most preferred embodiment a w/o emulsions according to the invention comprises 0.5 % w/w of the polymeric emulsifier according to the invention. If necessary, other emulsifiers may also be used in addition to the polymeric emulsifier according to the invention in the w/o emulsion according to the invention.

The w/o emulsions according to the invention may comprise 30 – 90%, preferably 35-60%, more preferably 40-60% by weight of oil.

Suitable oils for use in a w/o emulsion according to the present invention are non-metabolisable oils, metabolisable oils and mixtures of metabolisable and non-metabolisable oils. Non metabolisable oils that can be used in the adjuvants according to the invention include but are not limited to mineral oils and paraffin oils.

Metabolisable oils according to the invention include but are not limited to vegetable oils, animal oils, natural hydrocarbons, metabolisable synthetic or semi-

CONFIRMATION COPY

synthetic oils (such as Miglyol and Cetiol), fatty acid esters of propylene glycol and C6 to C24 fatty acids such as oleyl oleates, diesters of capric- or caprylic acids and the like. Suitable vegetable oils are peanut oil, soybean oil, sunflower oil, and the like. Suitable animal oils are squalane and squalene and the like.

Preferably the oil phase is metabolisable oil or a mixture of metabolisable oils, since non-metabolisable oils (mineral oils) tend to give local reactions at the injection sites. Preferred oils are the semi-synthetic oils such as Mygliol and Cetiol and oleyl oleates, esters of oleic ester, preferably ethyloleate.

With the adjuvants according to the invention very low viscosities can be reached and the emulsions are stable, while good adjuvant activity is obtained and no local reactions at the injection site occur.

The adjuvant according to the invention preferably comprises w/o emulsions having a viscosity below 450 mPa.s, preferably less than 250 mPa.s, more preferably less than 100 mPa.s. when tested in a Brookfield DV-I+ viscometer utilising spindle type No. 62 for 30 sec. at 60 r.p.m.

The aqueous phase of the w/o emulsions according to the present invention is usually made up of water, saline or buffer, such as phosphate buffered saline.

The adjuvant according to the invention can be used in vaccines. The vaccine will usually comprise antigenic material of an infectious agent. Vaccines comprising said adjuvants and an antigenic component derived from an infectious agent are likewise part of the present invention. Vaccines according to the invention may comprise the antigenic component in the discontinuous aqueous phase of the emulsion.

The use of a polymeric emulsifier which is a block copolymer having a general formula A-COO-B-OOC-A, in which B is the divalent residue of a water-soluble polyalkylene glycol and A is the residue of a oil-soluble complex monocarboxylic acid in the preparation of an adjuvant for use in a vaccine, and the use of these emulsifiers in vaccines is likewise part of the present invention.

Trough vaccination the immune system is triggered, resulting in a protective immune response against the infectious agent. Vaccines may be based on living, attenuated microorganisms, or killed (inactivated) microorganisms, as well as on subunits of microorganisms as antigenic component. Especially in the case of inactivated or sub-unit vaccines, an adjuvant is used to increase the immune response.

The antigenic material may be mixed with the adjuvant. In the vaccine the antigenic material may be present in the discontinuous water phase of the adjuvant as added. The vaccine preparations based on an adjuvant according to the invention can be prepared according to methods known in the art.

CONFIRMATION COPY

The w/o emulsions for use in the adjuvants according to the present invention can be used to prepare water-in-oil-in-water (w/o/w) emulsions based on droplets of the aforementioned w/o emulsion in an external aqueous phase. The w/o and w/o/w emulsions according to the present invention are suitable for use as adjuvant in vaccines, especially veterinary vaccines. Moreover, the w/o emulsions according to the present invention are suitable for use as vehicle for therapeutical agents, especially water-insoluble or water-sensitive active ingredients, and nutritious supplements. Thus in a further aspect the present invention provides for pharmaceutical compositions based on a w/o- or w/o/w emulsion according to the present invention.

The emulsions according to the invention can be prepared using standard techniques. In general the aqueous phase, the oil phase, the polymeric emulsifier according to the invention and optionally other emulsifiers are brought together and emulsified until a stable emulsion having the desired low viscosity is obtained. When emulsions are prepared, energy must be expended to form an interface between the oily and aqueous phases. Therefore, emulsification equipment includes a wide variety of agitators, homogenisers, colloid mills, jet mixers and ultrasonic devices. Production-size agitators can be propeller shaped or paddle shaped stirring systems, with rotation speed usually up to 2000 r.p.m., that are considered as low shear mixing procedures. Another type of production-site agitator is the colloid mill. The principle of operation of the colloid mill is the passage of the mixed phases of an emulsion formula between a stator and a high-speed rotor revolving at speeds of 2000 to 18000 rpm that is considered as a high shear mixing procedure.

Water-in-oil emulsions can be processed into a water-in-oil-in-water emulsion (also called "double emulsion"), where the internal and external aqueous phases are separated by an oil phase. This process consists of mixing the water-in-oil emulsion into an aqueous phase containing the proper emulsifying agent. In these systems both hydrophobic and hydrophilic emulsifier are used to stabilize the double emulsion.

The polymeric emulsifier according to the invention is preferably dissolved in the oil phase. Additional emulsifiers may be incorporated in the aqueous phase or oil phase.

In case of a w/o/w emulsion, a w/o emulsion according to the invention is prepared as primary w/o emulsion, which is subsequently added to a second aqueous phase and a second emulsifier and homogenised to obtain the desired w/o/w emulsion. The second emulsifier required to make the w/o/w emulsion is preferably an emulsifier with an HLB of 10-18, or a combination of two or more emulsifiers so that a desired HLB is obtained. Details concerning the manufacture of pharmaceutical emulsions can be found, for example in: "The Theory And Practice Of Industrial Pharmacy" (Eds: Lachman L. et al, Lea & Febiger, Philadelphia, USA, 1970, Chapter 16), and "Remington's

Pharmaceutical Sciences" (Eds: Gennaro, A.R. Mack Publishing Company, Easton, USA, 1990, 18th edition).

The vaccines according to the invention are preferably administered parenteral, e.g. intramuscularly, subcutaneous or intravenous. However if necessary the vaccines can also be administered non-parental e.g. per os, spraying, i.o. drops or intranasal drops. The low viscosities of the emulsions used in the adjuvants and/or vaccines of the present invention are extremely suitable for parenteral administration.

The following examples are merely to demonstrate the invention without limiting the invention to the particular embodiments.

EXAMPLES

Example 1

A water-in-oil emulsion containing the inactivated antigens of Infectious Bronchitis virus, strain Massachusetts and New Castle Disease virus, strain clone 30 are manufactured with a water/oil ratio of 50/50 % w/w. Besides the antigens, the water phase contains 0.01 m PBS. The organic phase contains a Medium Chained Triglyceride (Miglyol 840) and 3 % w/w PEG-30 Dipolyhydroxystearate (Arlacel P135) as emulsifier. Arlacel P135 is dissolved in Miglyol 840 at 60 °C while stirring. After dissolution this oil phase is cooled down to room temperature. The water phase is added slowly to this oil phase under high shear homogenisation using an Ultra Turrax type of homogeniser. The resulting water droplets have a size of mainly 1 µm. The viscosity of this emulsion is 115 mPa.s at 25 °C and showed sufficient stability in an accelerated stability test based on storage at 37 °C during three weeks.

Example 2

A water-in-oil emulsion containing the inactivated antigens of Infectious Bronchitis virus, strain Massachusetts and New Castle Disease virus, strain clone 30 are manufactured with a water/oil ratio of 70/30 % w/w. Besides the antigens, the water phase contains 0.01 m PBS. The organic phase contains a Medium Chained Triglyceride (Miglyol 840) and 3 % w/w PEG-30 Dipolyhydroxystearate (Arlacel P135) as emulsifier. Arlacel P135 is dissolved in Miglyol 840 at 60 °C while stirring. After dissolution this oil phase is cooled down to room temperature. The water phase is added slowly to this oil phase while stirring at 1100 r.p.m. using an IKA Eurostar mixer. The resulting water droplets have a size of mainly between 1 and 5 µm. The viscosity of this emulsion is 424 mPa.s at 25 °C and showed sufficient stability in an accelerated stability test based on storage

at 37 °C during three weeks. Three-week-old SPF female chicken, vaccinated intramuscularly once with 0.5 ml of this vaccine showed a mean 2log HI-IBV serum titre of 7.0 and a mean 2log HI-NDV serum titre of 4.4, nine weeks after vaccination.

Example 3

A water-in-oil-in-water emulsion is prepared using as emulsifiers: PEG-30 Dipolyhydroxystearate (Arlacel P135) and Synperonic F127. The primary water-in-oil emulsion is based on a water/oil ratio of 60/40 % w/w. The water phase contains the inactivated antigens of Infectious Bronchitis virus, strain Massachusetts and New Castle Disease virus, strain clone 30. Besides the antigens, the water phase contains 0.01 m PBS. The organic phase contains a Medium Chained Triglyceride (Miglyol 840) and 3 % w/w Arlacel P135 as emulsifier. Arlacel P135 is dissolved in Miglyol 840 at 60 °C while stirring. After dissolution this oil phase is cooled down to room temperature. The water phase is added slowly to this oil phase under high shear homogenisation using an Ultra Turrax type of homogeniser. The resulting water droplets have a size of mainly between 1 and 5 µm. The secondary emulsion is based on a water-in-oil/water of 60/40 % w/w. The external water phase contains besides 0.01 m PBS also 3 % w/w Synperonic F127. The water-in-oil emulsion is added slowly to this water phase under mediate shear (16.000 r.p.m.) homogenisation using an Ultra Turrax type of homogeniser. The resulting water-in-oil droplets have a size of mainly between 1 and 5 µm. The viscosity of this W/O/W emulsion is 110 mPa.s at 25 °C and showed stability for at least 6 months at 2 – 8 °C.

Example 4

A water-in-oil emulsion containing the inactivated antigens of Infectious Bronchitis virus, strain Massachusetts and New Castle Disease virus, strain clone 30, is manufactured with a water/oil ratio of 40/60 % w/w. Besides the antigens, the water phase contains 0.01 m PBS. The organic phase contains Ethyloleate and 0.1 % w/w PEG-30 Dipolyhydroxystearate (Arlacel P135) as emulsifier. Arlacel P135 is dissolved in at 60 °C while stirring. After dissolution this oil phase is cooled down to room temperature. The water phase is mixed into this oil phase while stirring at 1300 r.p.m. during 5 minutes using an IKA Eurostar mixer. The resulting water droplets have a size of mainly between 1 and 5 µm. The viscosity of this emulsion is 25 mPa.s at 25 °C and showed sufficient stability in an accelerated stability test based on storage at 37 °C during three weeks.

Example 5: water-in-oil formulation with low amounts of Arlacel P135

A water-in-oil emulsion is manufactured with a water/oil ratio of 60/40 % w/w. The water phase contains 0.01 m isotonic Phosphate buffer. The organic phase contains a Medium Chained Triglyceride (Miglyol 840) and 0.5 % w/w PEG-30 Dipolyhydroxystearate (Arlacel P135) as emulsifier. Arlacel P135 is dissolved in Miglyol 840 at 60 °C while stirring. After dissolution this oil phase is cooled down to room temperature. The water phase is added to this oil phase while stirring at 1100 r.p.m. during 5 minutes using an IKA Eurostar mixer. The resulting water droplets have a size of mainly between 1 and 5 µm. The viscosity of this emulsion is 127 mPa.s at 25 °C and showed sufficient stability in an accelerated stability test based on storage at 37 °C during three weeks.

Example 6

A water-in-oil emulsion containing the inactivated antigens of Infectious Bronchitis virus, strain Massachusetts and New Castle Disease virus, strain clone 30 is manufactured with a water/oil ratio of 40/60 % w/w. Besides the antigens, the water phase contains 0.01 m PBS. The organic phase contains Propyleneglycol Dicaprylcaprate and 0.1 % w/w PEG-30 Dipolyhydroxy-stearate (Arlacel P135) as emulsifier. Arlacel P135 is dissolved in Propyleneglycol Dicaprylcaprate at 60 °C while stirring. After dissolution this oil phase is cooled down to room temperature. The water phase is mixed into this oil phase while stirring at 1300 r.p.m. during 5 minutes using an IKA Eurostar mixer. The resulting water droplets have a size of mainly between 1 and 5 µm. The viscosity of this emulsion is 35 mPa.s at 25 °C and showed sufficient stability in an accelerated stability test based on storage at 37 °C during three weeks.

Example 7: Variation types of oil**Materials**

Mineral Oil (Marcol 52 from Exxon, USA), Eutanol G (Fatty alcohol from Henkel, Germany), Cetiol PGL (Hexyldecanol / Hexyldecyl Laurate from Henkel, Germany) and Isopropylmyristate (Merck, Germany), Estol 1526 (Medium Chained Triglyceride from Unichema, Spain) and Ethyloleate (AKZO-Nobel Chemicals, Sweden) are the types of oil tested. The ethyloleate and Estol 1526 used in this example, and in the following examples 8 and 9, contained 7.5% w/w vitamin E acetate. Arlacel P135 (ICI, UK) in a concentration of 0.25 % w/w (unless otherwise indicated) was used as surfactant. Arlacel P 135 was mixed into the oil phase while heating it up to 60 °C.

Type of formalin-inactivated viral avian antigen tested: New Castle Disease Virus (NDV), strain Clone 30 (produced in eggs). The total concentration of virus suspension used was 8 % w/v of the final vaccine. The aqueous phase, containing the virus suspension was diluted with 0.01 M Phosphate buffer, pH =7.2.

Preparation of the emulsions

Two types of preparations were tested. For low shear emulsions mixing of the aqueous phase into the oil phase was performed at 1100 - 1300 r.p.m. using the Eurostar mixer (IKA, Germany) provided with a propeller blade. For high shear emulsions, mixing was performed with high shear forces at 20.000 r.p.m. using the Ultra Turrax Type T25 (IKA, Germany). Variations of the water/oil ratios tested were 60/40 and 30/70 (all in % w/w). The droplet size of the emulsion was determined using interference microscopy at 1000x magnification (Olympus, model BX50, Japan). Droplet size of the emulsions prepared under low shear conditions were mainly 1 – 5 µm, while emulsions prepared under high shear conditions were mainly about 1 µm. Accelerated physical stability tests were performed by determining the appearance after storing the emulsions at 37 °C during three weeks. None of the samples tested showed emulsion breakdown after three weeks at 37 °C.

Immunization of animals

Groups of three week-old specific pathogen free (SPF) female chickens (n = 8 –10) were vaccinated intramuscularly with 0.5 ml of the vaccines, in the breast muscle. Blood samples for serological tests were taken on weeks 3, 6, 9 and 12 after vaccination. Virus haemagglutination inhibition (HI) tests for ND were performed to determine the levels of antiviral serum antibody titers.

Serum levels of NDV-specific antibodies were determined by haemagglutination inhibition assay. Serial two-fold serum dilutions were prepared in microtiter plates and mixed with an equal volume containing 8 haemagglutinating units / 50 µl NDV antigen. Titers were expressed as the reciprocal of the highest dilution that gives complete inhibition of haemagglutination. Samples were regarded to be positive at an inhibition of haemagglutination at dilution $\geq 1: 2$.

Results are shown in the following table.

CONFIRMATION COPY

Results

These emulsions were used for in-ovo vaccination. All the emulsions showed acceptable injectability. Also the results of the hatchability test were acceptable (between 80 and 95 %).

CONFIRMATION COPY

Claims

1. Adjuvant, for use in vaccine formulation, comprising a water-in-oil emulsion, characterised in that said emulsion comprises a polymeric emulsifier which is a block copolymer having a general formula A-COO-B-OOC-A, in which component B is the divalent residue of a water-soluble polyalkylene glycol and components A are the residue of a oil-soluble complex monocarboxylic acid.
2. Adjuvant according to claim 1, characterised in that components A have a molecular weight of at least 500 and component B has a molecular weight of at least 500.
3. Adjuvant according to claim 1 or 2, characterised in that component B is derived from polyethylene glycol and components A are derived from polyhydroxystearic acid, preferably from poly(12-hydroxy-stearic acid).
4. Adjuvant according to any of claims 1-3, characterised in that the polymeric emulsifier is Arlacel P135 or ATLOX 4912.
5. Adjuvant according to any of claims 1-4, characterised in that the emulsion comprises 0.01 – 15% w/w of the polymeric emulsifier.
6. Adjuvant according to claim 5, characterised in that the emulsion comprises 0.3 – 0.5% w/w of the polymeric emulsifier.
7. Adjuvant according to any of claims 1-6, characterised in that the oil is a metabolisable oil.
8. Adjuvant according to claim 7, characterised in that the oil is a semi-synthetic oil, or oleyl oleate.
9. Adjuvant for use in vaccine formulation, comprising a water-in-oil-in water (w/o/w) emulsion, characterised in that said (w/o/w) emulsion is based on a water in oil emulsion comprising a polymeric emulsifier which is a block copolymer having a general formula A-COO-B-OOC-A, in which component B is the divalent residue of a water-soluble polyalkylene glycol and components A are the residue of a oil-soluble complex monocarboxylic acid.
10. Vaccine comprising an adjuvant according to any of claims 1-9 and an antigenic component derived from an infectious agent.

CONFIRMATION COPY

11. Vaccine according to claim 10 wherein the antigenic component is comprised in the discontinuous aqueous phase of the emulsion.
12. Use of a polymeric emulsifier comprising a block copolymer having a general formula A-COO-B-OOC-A, in which component B is the divalent residue of a water-soluble polyalkylene glycol and components A are the residue of a oil-soluble complex monocarboxylic acid, in the preparation of an adjuvant.

CONFIRMATION COPY

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/EP 02/02145

A. CLASSIFICATION OF SUBJECT MATTER IPC 7 A61K9/107 A61K47/14 A61K39/39 B01F17/34		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) IPC 7 A61K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) INSPEC, WPI Data, EPO-Internal, PAJ, BIOSIS, MEDLINE, CHEM ABS Data, EMBASE, PASCAL		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00 33806 A (WETZEL THIERRY ; DEDEREN CHRISTIAN JOSEPH (BE); ICI PLC (GB); SERRI) 15 June 2000 (2000-06-15) example 26 ---	1-5, 9, 12
X	US 6 099 829 A (SCHEHLMANN VOLKER ET AL) 8 August 2000 (2000-08-08) examples ---	1-8, 12
Y	COX, JOHN C. ET AL: "Adjuvants - a classification and review of their modes of action" VACCINE (1997), 15(3), 248-256 , XP002201096 page 250, right-hand column, last paragraph -page 251, left-hand column, paragraph FIRST --- -/--	10
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents :		
A document defining the general state of the art which is not considered to be of particular relevance *E* earlier document but published on or after the international filing date *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed		*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. *Z* document member of the same patent family
Date of the actual completion of the international search 4 June 2002		Date of mailing of the international search report 19/06/2002
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer Zimmer, B

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP-02/02145

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
------------	--	-----------------------

Y	<p>TADROS T F ET AL: "STABILE W/O- UND W/O/W-EMULSIONEN MIT POLYMEREN TENSIDEN" PARFUMERIE UND KOSMETIK, HUETHIG, HEIDELBERG, DE, vol. 78, no. 4, April 1997 (1997-04), pages 30-34, XP000986652 ISSN: 0031-1952 page 34, left-hand column abstract</p>	10, 11
---	--	--------

Y	<p>-----</p> <p>GUPTA R K ET AL: "Adjuvants for human vaccines---current status, problems and future prospects" VACCINE, BUTTERWORTH SCIENTIFIC, GUILDFORD, GB, vol. 13, no. 14, 1 October 1995 (1995-10-01), pages 1263-1276, XP004057427 ISSN: 0264-410X page 1270, left-hand column, last paragraph</p> <p style="text-align: center;">-----</p>	11
---	---	----

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 02/02145

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0033806	A	15-06-2000	AU 1288600 A 26-06-2000
		BR 9915963 A 21-08-2001	
		CN 1333676 T 30-01-2002	
		EP 1137396 A1 04-10-2001	
		WO 0033806 A1 15-06-2000	
US 6099829	A	08-08-2000	DE 19749618 A1 12-05-1999
			EP 0917869 A2 26-05-1999
			JP 11209232 A 03-08-1999
			US 6197283 B1 06-03-2001

HIS PAGE BLANK (USPTO)

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☒ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

THIS PAGE BLANK (USPTO)